AJRI 2005; 53: 54–64

American Journal of Reproductive Immunology

Changes in Immune Cell Distribution and IL-10 Production are Regulated through Endometrial IP-10 Expression in the Goat Uterus

Imakawa K, Nagaoka K, Nojima H, Hara Y, Christenson RK. Changes in immune cell distribution and IL-10 production are regulated through endometrial IP-10 expression in the goat uterus. AJRI 2005; 53:54–64

PROBLEM: Changes in distribution or redistribution of immune cells are required for the establishment and maintenance of pregnancy, but these changes during early pregnancy have been poorly understood in the ruminant ungulates. Expression of a chemokine, interferon-γ (IFN-γ)-inducible protein 10 kDa (IP-10, CXCL10), was identified in the endometrium of pregnant goats. Population and/or distribution of endometrial immune cells and their cytokine productions could be regulated by IP-10 during the period of pregnancy establishment.
METHOD OF STUDY: Using reverse transcriptase-polymerase chain reaction (RT-PCR), expression of IP-10, IFN-γ, tumor necrosis factor-α, interleukin-10 (IL-10), CXCR3 mRNA and leukocyte cell surface markers, CD4, CD8, CD11b and CD45 mRNA during the caprine early pregnancy was investigated. The ability of IP-10 to stimulate peripheral blood mononuclear cells (PBMCs) migration was demonstrated using a chemotaxis assay. Changes in migration of PBMCs' immune cell population and cytokine expressions with IP-10 stimulation were investigated using flow cytometry and RT-PCR respectively.
RESULTS: Levels of IP-10, IL-10, CD4 and CD11b mRNA, and the number of CD4 and CD11b positive cells in pregnant goat endometrium were higher than those of cyclic goat endometrium. Migration of PBMCs was stimulated by recombinant caprine IP-10, and the effect was significantly reduced by neutralization with the use of an anti-IP-10 antibody. In the flow cytometric and RT-PCR analyses, migrated cells stimulated by IP-10 increased the expression of IL-10 and CD11b mRNA. Furthermore, IP-10 could stimulate the expression of IL-10 mRNA from PBMCs.
CONCLUSION: Endometrial chemokine IP-10 could regulate IL-10 production by resident and possibly migrated cells expressing CD11b, probably natural killer cells, and these changes may result in immune environments of the uterus suitable for conceptus implantation in ruminants.

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Key words: Caprine, early pregnancy, inducible protein-10, interleukin-10, migration, natural killer cell

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Submitted May 17, 2004;
revised November 15, 2004;
accepted November 29, 2004.

INTRODUCTION

In addition to biochemical communications, an interaction between the conceptus and maternal immune system may also be important in the establishment of pregnancy. Maternal immune system is highly complex and must be tightly regulated to elicit an effective and appropriate response to the conceptus. In ruminant ungulates, high incidences of conceptus losses, which may result from insufficient cellular and biochemical

interactions, occur during early pregnancy.[1,2] Such losses may also result from a lack of suitable changes in local immune environments, i.e. types and number of immune cells at or near implantation sites. Although exact mechanisms by which the maternal immune environments are established during the period of early pregnancy are not fully understood, it is likely that changes in the distribution of immune cells[3] and patterns of local cytokine productions are involved in the establishment of proper microenvironments *in utero*.[4–7]

Cytokines are soluble factors functioning in the immune systems, and acting as mediators for communication networks between cells of both lymphoid and non-lymphoid origins. Such molecules exert pleiotropic effects on these cells such as regulation of migration, activation, proliferation and possibly antibody production. These cytokines are functionally divided into proinflammatory cytokines, such as tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ), and regulatory cytokines such as interleukin-10 (IL-10) and transforming growth factor-β. It is believed that the local immunoregulation during early pregnancy is biased toward the increased production of regulatory cytokines, which suppress the proinflammatory reactions.[8,9] One of regulatory cytokines, IL-10, is thought to be produced by T helper 2 (Th2) lymphocytes; however, recent studies have shown that natural killer (NK) cells are also capable of producing IL-10.[10,11] In humans and mice, the redistribution of immune cells during early pregnancy is considered prerequisite for successful implantation, particularly increases in the number of uterine NK (uNK) cells at the implantation site.[12,13] These uNK cells are thought to produce IL-10, which in turn alters uterine environments suitable for embryonic survival. In ruminant ungulates, NK-like cells with lytic activity were found in the ovine endometrium during early pregnancy.[14] It is therefore suspected that uNK or NK-like cells also play an important role during the period of implantation in ruminants. However, biochemical and molecular mechanisms by which these cells migrate and/or are recruited to an implantation site have not been well characterized.

In ruminants, a major protein, IFN-τ, produced by the developing blastocyst is implicated in the process of maternal recognition of pregnancy.[15–17] IFN-τ prevents the regression of corpus luteum (CL), at least in part by inhibiting endometrial estrogen receptor expression, thus preventing estrogen stimulation of the oxytocin receptor, which impedes pulsatile releases of endometrial luteolysin, prostaglandin $F_{2\alpha}$.[18] Furthermore, IFN-τ is known to regulate lymphocyte proliferation[19–21] and cytokine production,[22,23] suggesting that IFN-τ may also play a role in the changes and/or adjustment of immunological environments suitable for the process of implantation. Recently, the endometrial expression of a chemokine, IFN-γ-inducible protein 10 kDa (IP-10, CXCL10), during the period of sheep and goat implantation was found in our laboratory. More importantly, maternal expression of IP-10 was stimulated by conceptus IFN-τ.[24,25] Chemokines are a family of structurally related cytokines with low molecular masses and play a key role in multiple aspects of inflammatory and immune responses by promoting migration and activation of different subpopulations of immune cells.[26] IP-10 is a member of the C-X-C chemokines and targets NK cells and Th1 lymphocytes through the C-X-C chemkine receptor 3 (CXCR3).[27,28] Because other chemokines are also expressed in the endometrium of ruminant ungulates,[29,30] it is suspected that local expressions of chemokines, which play a role in the distribution or redistribution of immune cells and their cytokine productions, are required for successful pregnancy.

The present study was undertaken to gain better insight into the distribution and/or redistribution of immune cells and subsequent changes in cytokine productions by one of endometrial chemokines, IP-10, in the goat uterus. Transcript changes in IP-10, IFN-γ, TNF-α, IL-10, CXCR3 and leukocytes' cell surface markers, CD4, CD8, CD11b and CD45 were studied during early pregnancy. Studies were extended to examine whether IP-10 could regulate the migration of peripheral blood mononuclear cells (PBMCs) and to investigate changes in cell phenotypes and/or expression of cytokine genes in the migrated PBMCs.

MATERIAL AND METHODS

Animals and Tissue Preparation

Shiba goats were maintained at the University of Tokyo farm and the protocol for goat experimentation has been reviewed and approved by the animal care committee at the University. Uteri from cyclic goats on day 15 ($n = 7$) and pregnant goats on days 17 ($n = 3$) and 20 ($n = 5$) were removed by hysterectomy under isoflurane anesthesia.[25] Two uteri each from day 15 cyclic goats and day 20 pregnant goats were frozen immediately for subsequent immunohistochemical studies. Endometrial tissues collected from goats ($n = 3$ for each day examined) were frozen immediately and stored at −70°C; these frozen tissues were subjected to RNA extraction. Whole blood was collected from day 15 cyclic goats, and PBMCs were used for IP-10 stimulation and chemotaxis assays.[25]

In Vitro Culture

The PBMCs were separated from ethylenediaminetetraacetic acid-treated whole blood (80 mL) by

density gradient centrifugation (800 × *g* at 20°C for 30 min, OptiPrep; Nycomed, Roskilde, Denmark) and were suspended in RPMI 1640 medium supplemented with 10% fetal calf serum (Sigma, St Louis, MO, USA), 40 U/mL of penicillin, 40 μg/mL of streptomycin and anti-pleuropneumonia-like organisms agent (Invitrogen Corp., Carlsbad, CA, USA). PBMCs counted and adjusted to 3×10^7 cells/mL were plated onto 6-well costar plates (3 mL per well), which were then incubated with 20 ng/mL recombinant caprine IP-10 (rcIP-10),[25] 10 ng/mL recombinant bovine IFN-τ (Katakura Industries Co., Tokyo, Japan) or control (20 ng/mL recombinant glutathione S-transferase, GST)[25] at 37°C, 5% CO_2 and 95% air atmosphere for 8 hr. After incubation, cells were immediately processed for total RNA extraction.

Chemotaxis Assay

Migration of PBMCs was assessed in a 96-well modified Boyden chamber (NeuroProbe, Cabin John, MD, USA) using polyvinylpyrrolidone-free polycarbonate membrane (5 μm pore size, NeuroProbe), as described previously.[25] In brief, isolated PBMCs were labeled with the intracellular fluorescent dye, calsein-AM (4 μM, Molecular Probes Inc., Eugene, OR, USA), at 37°C for 30 min. Dulbecco's modified Eagle's medium (DMEM)-0.1% bovine serum albumin (BSA) (without phenol red) supplemented with 20 ng/mL rcIP-10 was added to the bottom wells of the chemotaxis chamber, whereas labeled PBMCs (5×10^6 cells/mL) in DMEM-0.1% BSA (without phenol red) were added to the top wells of the chamber. After the chambers were incubated at 37°C and 5% CO_2 atmosphere for 2 hr, fluorescence of cells in the bottom wells was measured using a fluorescence reader (excitation filter 485 nm and emission filter 535 nm, ARVO SX 1420 Multilabel Counter, Perkin-Elmer Life Sciences Inc., Boston, MA, USA). For the blocking experiments, rcIP-10 was pre-incubated at 37°C for 1 hr with 30 μg/mL of a rabbit polyclonal antibody to rcIP-10 or control rabbit IgG (Sigma) before addition to the bottom chamber.[25] Three replicates were performed for each treatment, and three independent experiments were carried out for each treatment.

Flow Cytometric Analysis

To determine cell phenotypes, migrated cells on the chemotaxis assay were subjected to flow cytometric analysis (fluorescence-activated cell sorter, FACS). Chemotaxis assay of non-fluorescent-labeled PBMCs was performed as aforementioned.[25] Migrated cells were collected and blocked with Block Ace (Dainippon Pharmaceutical, Osaka, Japan) at 4°C for 30 min to prevent non-specific binding of antibodies, and then incubated with 1:10 dilution of monoclonal antibodies to CD4, CD8 (generously provided by Dr Barcham, The University of Melbourne) or CD11b (Serotec Inc., Raleigh, NC, USA) at 4°C for 1 hr. After the incubation, cells were washed in cold phosphate-buffered saline, and incubated with flourescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (15 μg/mL, Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA) at 4°C for 30 min. Flow cytometric analyses were performed using a FACS Calibur cytofluorimeter (Becton Dickinson, Mountain View, CA, USA).

Immunohistochemical Analysis

Frozen whole uteri sections (10 μm) were mounted onto silane-coated slides and fixed in acetone. Non-specific binding was blocked by 1-hr treatment with Block Ace at room temperature, followed by incubation with monoclonal antibodies to CD4, CD8 or CD11b at 4°C for 12 hr. Slides were then incubated with biotin-conjugated donkey anti-mouse IgG (1:400, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) at room temperature for 1 hr. Immunoreactions were visualized by diaminobenzidine (Sigma). An equal concentration of normal mouse IgG was used on semi-serial sections of the same tissue to evaluate non-specific binding (negative control). Tissue sections were then counterstained with hematoxylin.

RT-PCR Analysis

Using Isogen (Nippon Gene, Tokyo, Japan), total RNAs were extracted from endometrial tissues and PBMCs.[24] Total RNA samples (2 μg) were first reverse transcribed with SuperScriptII (Invitrogen) and oligo-dT primers (20 μL reaction volume).[25] Relative mRNA levels of IP-10, CXCR3, IL-10, IFN-γ, TNF-α, CD4, CD8, CD11b, CD45 and glyceraldehyde 3-phosphate dehydrogenase (G3PDH) were determined from PCR amplification using oligonucleotide primers (Table I). Each reaction consisting of primer pairs was run with RT template (1 μL) and AmpliTaq Gold (0.625 U; Roche Molecular Systems, Branchburg, NJ, USA) in a final volume of 25 μL. All PCR reactions consisted of 35 or 40 cycles at 95°C for 1 min, 60°C for 1 min and 72°C for 1 min, followed by the final extension at 72°C for 12 min. Following agarose gel electrophoresis and visualization with ethidium bromide, PCR products were analyzed using an image analysis system (ATTO Corporation, Tokyo, Japan) equipped with the Quantity One (v3.0 software; PDI, Inc., Huntington Station, NY, USA). The identity of each amplified PCR product was verified by sequence analysis using a Perkin-Elmer sequencer (model ABI Prism 377 XL, Roche) after cloning into the pGEM-T Easy vector (Promega, Madison, WI, USA).

TABLE I. Oligonucleotide Primers Used for PCR Reactions

Name	Sequence of forward and reverse primer	No. of cycles	Product size (bp)
IP-10	5′-CAAGGCATACCTCTCTCTAG-3′ 5′-TTCAGACATCTTTTCTCCCC-3′	40	170
CXCR3	5′-GCATCAGCTTCGATCGGTAC-3′ 5′-GATGCGGGCGTAGCAATAGG-3′	40	284
IL-10	5′-CTGTCTGACAGCAGCTGTAC-3′ 5′-ATACGTCACAGAGAAGCTCA-3′	40	646
IFN-γ	5′-CGATGAAATACACAAGCTCC-3′ 5′-GATTACATTGATGCTCTCCG-3′	40	504
TNF-α	5′-ACAACGGGCCACCAACCATC-3′ 5′-TCTCCCAGGACACCTTGACC-3′	40	360
CD4	5′-GTACTGAGGCAAGGTCCGCA-3′ 5′-TGCACTATCTCCCAGAGCCA-3′	35	320
CD8	5′-ACCCTGAGAAACTTCCGCCA-3′ 5′-TGACCAATGAGAGGAGAAGG-3′	35	310
CD11b	5′-TCCTTTGCTGGGTGCTGTGG-3′ 5′-GCAGGACAGCATCACACTTC-3′	35	440
CD45	5′-TTCTGCAACTGCTCCCTCGA-3′ 5′-AATGGAGGCTCACATGAAGG-3′	35	240
G3PDH	5′-ATGGGGAAGGTGAAGGTCGG-3′ 5′-ATCATATTGGAACATGTAAA-3′	35	150

Nucleotide sequence comparisons were performed using BLAST network program (National Center for Biotechnology Information, NIH, Bethesda, MD, USA).

Statistical Analysis

Light intensity (reverse transcriptase-polymerase chain reaction, RT-PCR) measurements were subjected to least squares (LS) ANOVA using the general linear model procedures of the Statistical Analysis System (version 6.0; SAS Institute, Cary, NC, USA). The model used in the LS-ANOVA included day (or treatment) and replicate as sources of variation. The light intensity from G3PDH PCR products was used as covariates for RT-PCR analyses. The least square mean (LSM) value and S.E. illustrated in the figures were derived from this analysis.

RESULTS

Expression of Various Cytokines in the Caprine Uterus

In agreement with our previous studies,[24,25] IP-10 mRNA expression was higher in the endometrium of pregnant goats than that of cyclic goats (Fig. 1). The expression of IP-10 receptor CXCR3 mRNA also increased in endometrium of pregnant goats as compared with endometrium of cyclic goats. Levels of IL-10, IFN-γ and TNF-α transcripts were also examined, and changes in IL-10 mRNA levels in endometrium of pregnant goats were found similar to those of IP-10 expression (Fig. 1). The presence of IFN-γ and TNF-α mRNA was detected in the endometrium of both cyclic and pregnant animals, but changes in mRNA levels of these cytokines were minimal.

Expression and Population of Immune Cells in the Caprine Uterus

To determine the distribution of immune cells in the pregnant uterus, the expression of CD4 (generally associated with Th lymphocytes), CD8 (usually associated with T cytotoxic lymphocytes), CD11b (NK cells and macrophages) and CD45 (all leukocytes) mRNA was determined using RT-PCR analysis. Levels of CD4 and CD11b mRNA were greater in endometrium of day 20 pregnant goats than endometrium from goats on other days examined; however, significant changes in CD8 and CD45 mRNA levels were not observed (Fig. 2).

Immunohistochemical analysis revealed that CD4, CD8 and CD11b positive cells were localized in cyclic and pregnant goat uteri (Fig. 3). Although a definitive conclusion could not be made, the number of CD4, CD8 and CD11b positive cells appeared to be higher in the subepithelial stroma and luminal epithelium of pregnant goats than those of cyclic goats. In addition, substantial numbers of positive cells were also

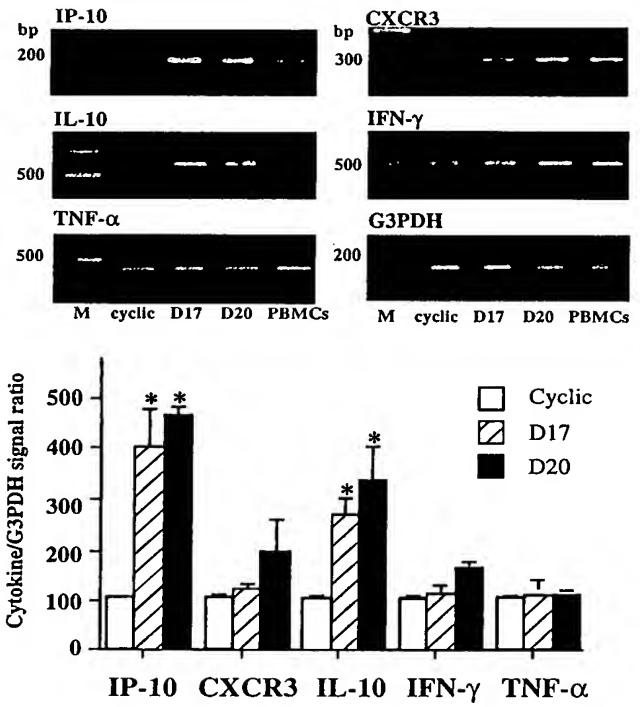

Fig. 1. Examination of chemokine and cytokine mRNA levels in the caprine endometrium. (Upper) RT-PCR analysis of changes in IP-10, CXCR3, IL-10, IFN-γ, TNF-α and G3PDH mRNA levels. Cyclic: day 15 cyclic goat endometrium ($n = 3$). D17 and D20: days 17 and 20 pregnant goat endometrium ($n = 3$ each). PBMCs: peripheral blood mononuclear cells from day 15 cyclic goats ($n = 3$). One set of representative data is shown. (Lower) Densitometric analysis of PCR products. Data represent those from three animals and each performed in triplicates. Bars represent LSM ± S.E., and differences in these mRNA levels within a treatment group relative to that in cyclic animals are shown with an asterisk ($P < 0.05$).

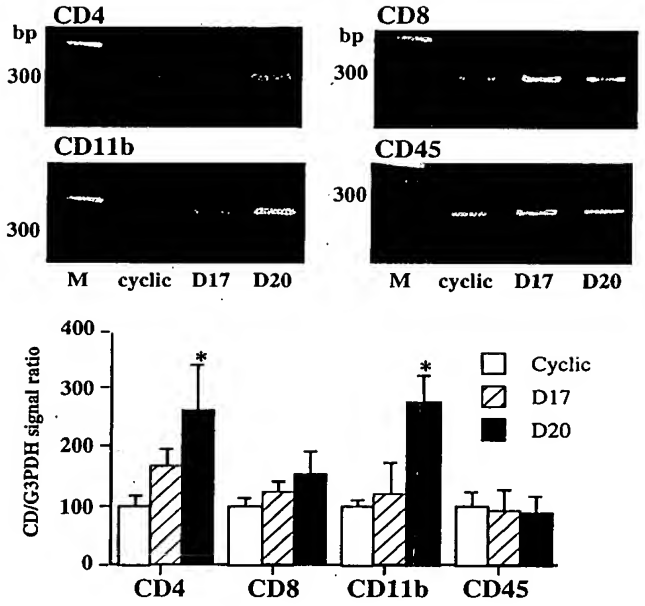

Fig. 2. Examination of CD4, CD8, CD11b and CD45 mRNA levels in the caprine endometrium. (Upper) RT-PCR analysis of expressions of CD4, CD8, CD11b and CD45 mRNA levels. Cyclic: endometrium from day 15 cyclic animals ($n = 3$). D17 and D20: endometrium from days 17 and 20 of pregnant animals ($n = 3$ each). One set of representative data is shown. (Lower) Densitometric analysis of PCR products. Data represent those from three animals and each performed in triplicates. Bars represent LSM ± S.E., and differences in these mRNA levels relative to that in cyclic animals are shown with an asterisk ($P < 0.05$).

localized on the surface of trophectoderm in pregnant goats.[25] In the cyclic goat uterus, CD4, CD8 and CD11b positive cells were rarely localized in the subepithelial stroma (Fig. 3).

Effect of IP-10 on Immune Cell Migration

To ascertain the types of immune cells that migrated in response to the IP-10 treatment, isolated PBMCs were subjected to chemotaxis assay, followed by flow cytometric analysis against anti-CD4, anti-CD8 or anti-CD11b antibody. Chemotaxis assay showed that IP-10 increased the migration of PBMCs and the effect of IP-10 was neutralized by pre-treatment with the anti-IP-10 antibody (Fig. 4A). Flow cytometric analysis revealed that the population of CD4 and CD8 positive cells did not change in migrated cells stimulated with IP-10. But, the number of CD11b positive cell migration, when PBMCs were treated with IP-10, was higher than that of a group of cells without IP-10 stimulation. These data indicate that IP-10 could stimulate the migration of CD11b positive cells rather than CD4 and CD8 positive cells (Fig. 4B). The expression of CD4, CD8, CD11b and CXCR3 mRNA in migrated PBMCs with or without IP-10 stimulation were examined using RT-PCR analysis (Fig. 5A,B). It was confirmed that IP-10-stimulated migrated cells expressed more CD11b and CXCR3 mRNAs than those in non-stimulated migrated cells. However, the expression of CD4 and CD8 mRNAs did not change in migrated PBMCs with or without IP-10 stimulation (Fig. 5A,B). These observations were consistent with the results obtained from the flow cytometric analysis.

Effect of IP-10 on Cytokine Expressions

Cells that migrated with IP-10 stimulation had increased the expression of IL-10 and IFN-γ mRNAs, but the degree of increase in IL-10 mRNA was greater than that in IFN-γ mRNA (Fig. 5A,C). The expression of TNF-α did not change in migrated PBMCs with or without IP-10 stimulation. To determine whether IP-10 and/or IFN-τ could directly stimulate IL-10 expression, the expression of IL-10,

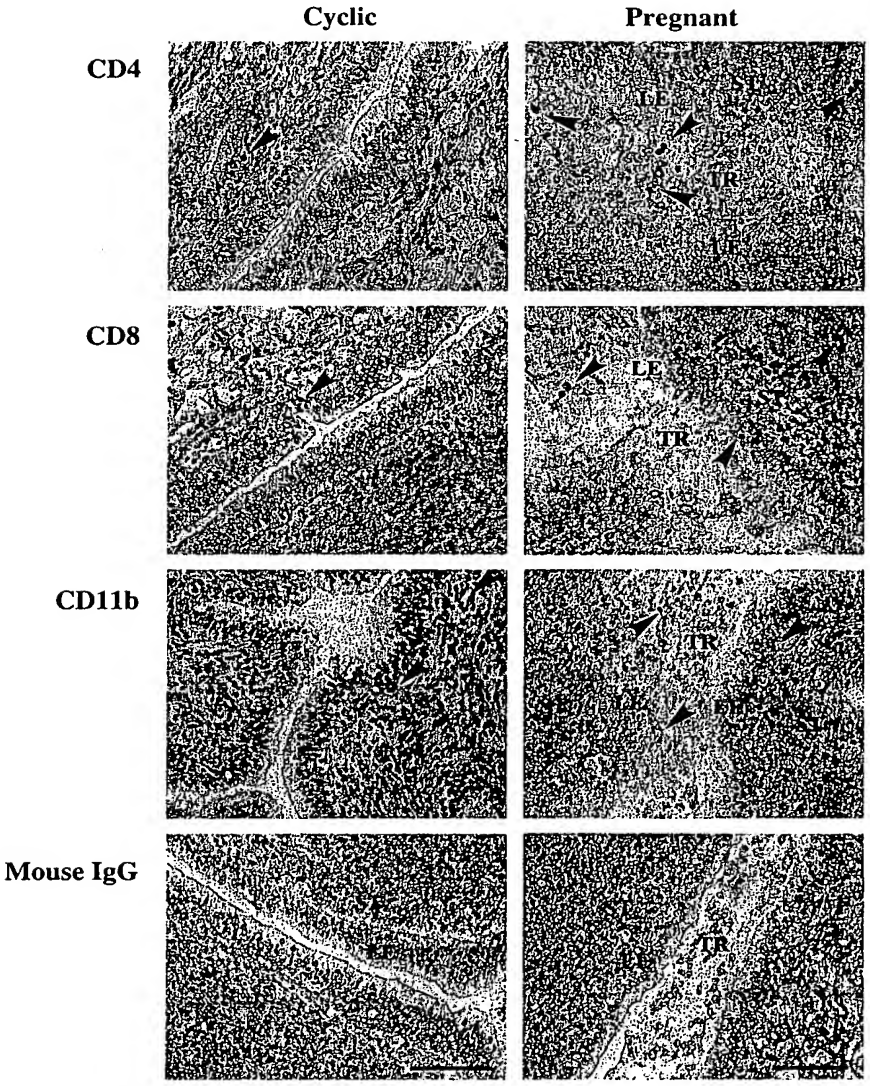

Fig. 3. Immunohistochemical detection of CD4, CD8 and CD11b in the cyclic (day 15) and pregnant (day 20) uteri. Antibodies used were anti-CD4, CD8 or CD11b mouse monoclonal antibody (all antibodies are cross-reactive to goats) and mouse IgG was used as a negative control. One set of representative data is shown. Arrowheads indicate positive signals. LE, luminal epithelium; ST, subepithelial stroma; TR, trophoblast. Bar = 100 μm.

IFN-γ and TNF-α in PBMCs stimulated by IP-10, IFN-τ or recombinant GST protein (control) was investigated (Fig. 6). The low endogenous expression of IL-10 mRNA in PBMCs was observed using RT-PCR analysis (Figs 1 and 6A). IL-10 mRNA expression was stimulated with IP-10 or IFN-τ treatment, but the stimulation of IL-10 by IP-10 was greater than that by IFN-τ (Fig. 6B). The expression of IFN-γ mRNA was also stimulated by IP-10, but TNF-α mRNA was not affected by any treatments.

DISCUSSION

In addition to the prevention of luteolysis, proper interaction or adjustment between the conceptus and maternal immune system is required for pregnancy establishment and maintenance. In ruminant ungulates, a conceptus factor IFN-τ is implicated in the process of maternal recognition of pregnancy by indirectly inhibiting CL regression.[15–18] This IFN also regulates lymphocyte proliferation and cytokine production.[19–23] Recent studies revealed that endometrial IP-10 production appeared to be regulated by conceptus IFN-τ, and the migration of PBMCs was affected by IP-10 expression.[24,25] Quite recently, the secretion of IP-10 and monokine induced by IFN-γ (MIG), both of which share the same receptor CXCR3, by cultured human endometrial stromal cells was found to be induced by progesterone, and endometrial IP-10 and MIG concentrations were correlated with the number of endometrial NK cells.[31] Based on these observations, progesterone, conceptus IFN-τ and endometrial chemokines were suspected to play an important role in the

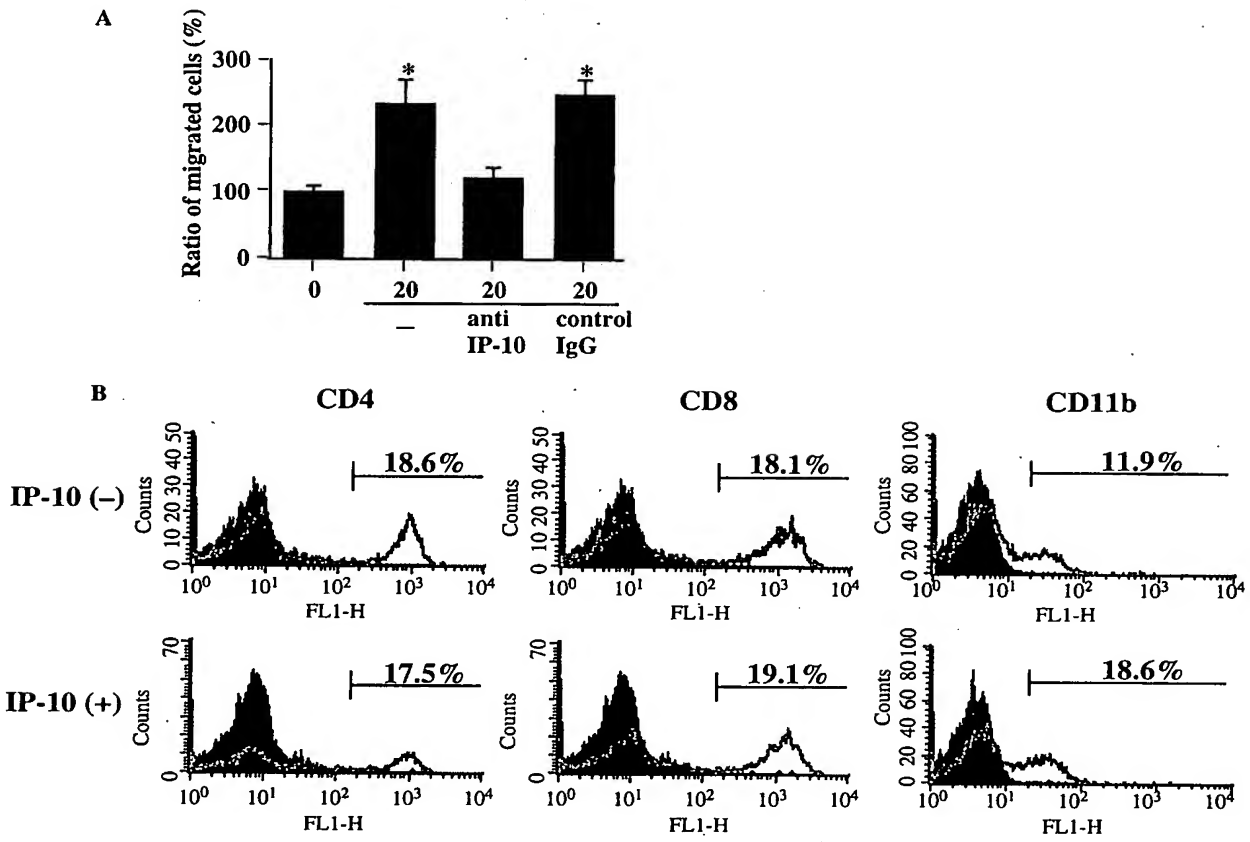

Fig. 4. Migration of PBMCs and changes in cell phenotypes with the IP-10 treatment. (A) Migration of PBMCs by IP-10. Effects of recombinant caprine IP-10 (20 ng/mL) on the migration of PBMCs pretreated with 30 μg/mL of anti-IP-10 IgG or normal rabbit IgG were tested using a Boyden chamber. Bars represent LSM ± S.E. An asterisk indicates a significant difference ($P < 0.05$). Three replicates were carried out for each treatment, and three independent experiments were performed for each treatment. (B) Expression of CD4, CD8 and CD11b cell markers on migrated PBMCs. Migrated cells in the presence or absence of IP-10 were harvested from three independent cultures. For flow cytometric (FACS) analysis, these cells were blocked against non-specific binding, and antibodies against CD4, CD8 and CD11b were then added to the cells. Cells reactive to cell marker antibody were identified using FITC-conjugated anti-mouse IgG. Numbers in figures indicate the percentage of positive cells. The experiment was performed three times independently.

immune cell distributions and patterns of cytokine productions at the endometrium, resulting in the adjustment and/or establishment of uterine environments suitable for the survival of ruminant conceptuses during early pregnancy.

In this study, the increase in IP-10 and IL-10 mRNA expression, but not IFN-γ or TNF-α was observed in the endometrium of pregnant goats. IL-10 is a cytokine with potent immunosuppressive effect,[32] and is considered to play a crucial role in the alteration of immune-associated environments *in utero* during early pregnancy.[32] It has been documented that regulatory cytokines including IL-10 are produced by Th2 lymphocytes and NK cells.[10,11,33–36] In addition, increase in CD4 (generally associated with Th1 and Th2 lymphocytes) and CD11b (associated with macrophages and NK cells) mRNA expression was realized in the endometrium of pregnant goats. These results indicated that endometrial IL-10 could be produced by Th2 lymphocytes and/or NK cells in the goat uterus. Furthermore, expressions of IP-10 and IL-10 in the pregnant endometrium appeared to be parallel, suggesting that endometrial IL-10 production could be increased with IP-10 stimulation. This notion was supported with the observations from chemotaxis assays in which PBMCs migrated with IP-10 stimulation had increased transcripts of IL-10, CXCR3 and CD11b, but not IFN-γ, TNF-α, CD4 or CD8. These results suggest that caprine IP-10 might affect migration or infiltration of NK cells rather than T lymphocytes or macrophages, which is consistent with the previous study in humans, in which IP-10 directed migration of NK cells by binding to and activating CXCR3 receptor.[27,31,37]

It has long been suspected that uNK cells are required for the regulation of uterine environments in

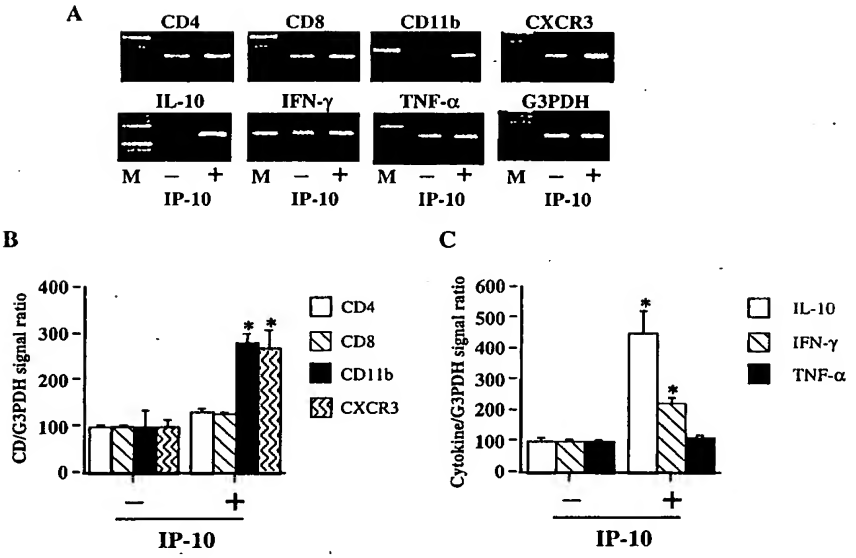

Fig. 5. Changes in cell surface marker and cytokine expressions on IP-10-stimulated migrated PBMCs or not migrated PBMCs. (A) Results from RT-PCR analysis for CD4, CD8, CD11b CXCR3, IL-10, IFN-γ, TNF-α and G3PDH mRNA levels on migrated PBMCs in the presence or absence of 20 ng/mL IP-10. One set of representative data is shown. (B) Densitometric analysis of RT-PCR products on CD4, CD8, CD11b cell surface markers, CXCR3 and G3PDH mRNA levels. Bars represent LSM ± S.E., and differences in these mRNA levels relative to that of PBMCs without IP-10 treatment are shown with an asterisk ($P < 0.05$). (C) Densitometric analysis of transcript levels resulted from RT-PCR studies on IL-10, IFN-γ, TNF-α and G3PDH mRNA levels. Bars represent LSM ± S.E., and differences in these mRNA levels relative to that of PBMCs without IP-10 treatment are shown with an asterisk ($P < 0.05$).

the human and murine pregnancy. When pregnancy occurs and advances, the number of uNK cells increases progressively. uNK cells are known to produce IFN-γ, which is involved in the local immunoregulation during early pregnancy.[38] In fact, the results from uNK cell-deficient mice reveal that IFN-γ concentrations at the implantation sites are considerably lower than those in wild-type mice, and deciduas of pregnant uterus exhibit necrosis, hypocellularity and abnormal vasculature.[38–40] It is well documented that IFN-γ production is required for the establishment of murine pregnancy;[38] however, this proinflammatory cytokine is detrimental to the maintenance of pregnancy if the production persists beyond pregnancy establishment periods. Thus, the proper shift of the proinflammatory to regulatory cytokine productions by uNK cells may determine the success or failure of implantation processes. In ruminants, data related to uNK cell distribution and to IFN-γ production in the uterus are limited, but shifts of uterine environments detrimental to suitable for conceptus survival are required for pregnancy establishment.

In experiments with CBA × DBA/2 mice (a spontaneous fetal resorption model), the intrauterine injection of recombinant ovine IFN-τ has reduced conceptus and embryo resorption.[41] It is thought that increased production of uterine IL-10 by IFN-τ administration is a reason for higher conceptus survival. In addition, intrauterine administration of anti-IFN-γ antibody to CBA × DBA/2 mice reduces the embryo resorption rate.[41] In the normal pregnancy model, CBA × BALB/c mice, endometrial IL-10 production is much higher than that in the CBA × DBA/2 mice, and when IL-10 is given to CBA × DBA/2 mice, embryo and fetal mortality decreases significantly.[41] The paradox in pregnancy immunology could be explained by one of the hypotheses that uNK cells in cyclic and pregnant endometrium produce both IFN-γ and IL-10, but when pregnancy occurs, uNK cells switch the cytokine production from IFN-γ to IL-10. In ruminants, it is possible that both conceptus factor IFN-τ and endometrial IP-10 have a role in the alteration of immune cell phenotypes toward uNK cells, which increase the production of IL-10 in the progesterone dominant-pregnant uterus.

In this study, IFN-τ as well as IP-10 stimulated the expression of IL-10 mRNA in PBMCs. The ability of IFN-τ to stimulate IL-10 production and to function as a immunosuppressor has been investigated previously.[41,42] IFN-τ promoted the proinflammatory to the regulatory cytokine productions; however, biochemical and/or molecular mechanisms regulating such productions have not been clarified. Moreover, the exact mechanism by which IL-10 production is stimulated by IFN-τ *in vivo* has not been determined. Co-expression

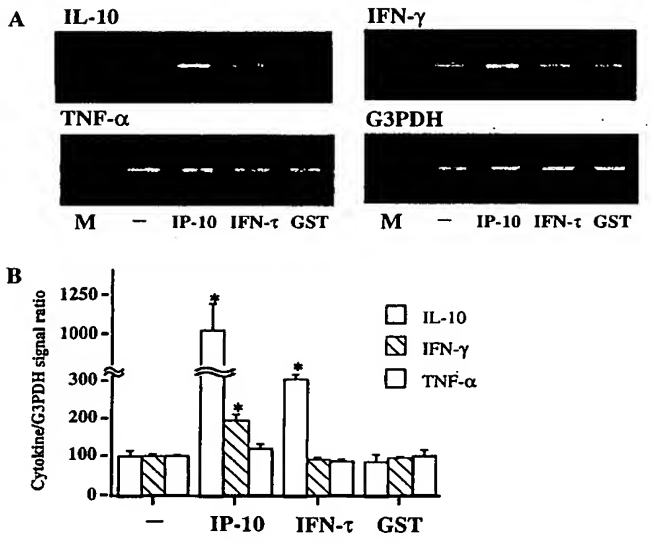

Fig. 6. Effect of IP-10 and IFN-τ on cytokine mRNA levels in PBMCs. (A) RT-PCR of IL-10, IFN-γ, TNF-α and G3PFH mRNA on PBMCs stimulated with 20 ng/mL IP-10, 10 ng/mL IFN-τ or control as 20 ng/mL recombinant glutathione S-transferase. One set of representative data is shown. (B) Densitometric analysis of RT-PCR products for several cytokines, IL-10, IFN-γ and TNF-α, mRNA and G3PDH mRNA. Bars represent LSM ± S.E., and differences in these mRNA levels relative to that of PBMCs without any treatment are shown with an asterisk ($P < 0.05$).

and interaction of IP-10 and IL-10 has been reported from the studies of antitumor immunotherapy, which reveals that IP-10 is up-regulated in IL-10 expressing tumors and is important in the tumor-inhibitory activity of IL-10.[43,44] In contrast, it was reported that IL-10 might act indirectly to suppress the expression of IP-10 from immune cells.[45,46] Although the expression and interactions of IP-10 and IL-10 are demonstrated in many experimental models of anti-viral and anti-tumor effects and/or successful implantation, further investigations are required to elucidate effects and regulatory mechanisms of these factors during the period of pregnancy establishment in ruminant ungulates.

CONCLUSION

The results from the present investigation showed the expression of IP-10, IFN-γ, TNF-α, IL-10, CXCR3 mRNA and leukocytes cell surface markers, CD4, CD8, CD11b and CD45 mRNA in the caprine uterus. Data revealed that IP-10, found in the endometrium, could regulate the migration of PBMCs expressing IL-10 and CD11b, probably NK cells. These observations suggest that endometrial IP-10, regulated by IFN-τ and possibly progesterone, plays an important role in the redistribution of uNK cells and/or the change of uNK cell phenotypes, which increase the production of IL-10. These changes in the local immune cell populations during the early pregnancy period play a crucial role for successful implantation in ruminants. Further investigations are required to better characterize and define a physiological role(s) for resident NK cells and NK cells recruited by endometrial IP-10 during early pregnant period in ruminants.

Acknowledgments

We thank Dr Garry Barcham (The University of Melbourne) for generously providing anti-ovine CD4 and CD8 monoclonal antibodies. We would like to thank Mr R. Sako and Drs Toru Sawasaki and Jun-Yu Li for their care and preparation of experimental animals at the Experimental Station for Bio-medical research, the University of Tokyo. We thank a PhD student, Tomohiro Yonezawa, for his assistance in anesthetic procedure during goat surgery. Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and do not imply recommendation or endorsement by the US Department of Agriculture. This work was supported by a Grant-in-Aid for Scientific Research (13556050 and 14206032) to KI from the Japan Society for the Promotion of Science. K. Nagaoka is supported by Research Fellowships of the Japan Society for the Promotion of Science for Young Scientists.

REFERENCES

1. Roberts RM, Farin CE, Cross JC: Trophoblast proteins and maternal recognition of pregnancy. Oxf Rev Reprod Biol 1990; 12:147–180.
2. Peters AR: Embryo mortality in the cow. Anim Breed Abstr 1996; 64:587–598.
3. Perez-Martinez M, Luna J, Mena R, Romano MC: Lymphocytes and T lymphocytes subsets are regionally distributed in the female goat reproductive tract: influence of the stage of oestrous cycle. Res Vet Sci 2002; 72:115–121.
4. Hansen PJ: Interactions between the immune system and the ruminant conceptus. J Reprod Fertil Suppl 1995; 49:69–82.
5. Engelhardt H, King GJ: Uterine natural killer cells in species with epitheliochorial placentation. Nat Immunol 1996–97; 15:53–69.
6. Martal J, Chene N, Camous S, Huynh L, Lantier F, Hermier P, L'Haridon R, Charpigny G, Charlier M, Chaouat G: Recent developments and potentialities for reducing embryo mortality in ruminants: the role of IFN-tau and other cytokines in early pregnancy. Reprod Fertil Dev 1997; 9:355–380.
7. Hansen TR, Austin KJ, Perry DJ, Pru JK, Teixeira MG, Johnson GA: Mechanism of action of interferon-tau in

the uterus during early pregnancy. J Reprod Fertil Suppl 1999; 54:329–339.
8. Wegmann TG, Lin H, Guilbert L, Mosmann TR: Bidirectional cytokine interactions in the maternal-fetal relationship: is successful pregnancy a Th2 phenomenon? Immunol Today 1993; 14:353–356.
9. Raghupathy R, Makhseed M, Azizieh F, Omu A, Gupta M, Farhat R: Cytokine production by maternal lymphocytes during normal human pregnancy and in unexplained recurrent spontaneous abortion. Hum Reprod 2000; 15:713–718.
10. Mehrotra PT, Donnelly RP, Wong S, Kanegane H, Geremew A, Mostowski HS, Furuke K, Siege JP, Bloom ET: Production of IL-10 by human natural killer cells stimulated with IL-2 and/or IL-12. J Immunol 1998; 160:2637–2644.
11. Veenstra van Nieuwenhoven AL, Bouman A, Moes H, Heineman MJ, de Leij LF, Santema J, Faas MM: Cytokine production in natural killer cells and lymphocytes in pregnant women compared with women in the follicular phase of the ovarian cycle. Fertil Steril 2002; 77:1032–1037.
12. Guimond MJ, Luross JA, Wang B, Terhorst C, Danial S, Croy BA: Absence of natural killer cells during murine pregnancy is associated with reproductive compromise in TgE26 mice. Biol Reprod 1997; 56:169–179.
13. King A: Uterine leukocytes and decidualization. Human Reprod Update 2000; 6:28–36.
14. Segerson EC, Beetham PK: High-density ovine endometrial cells exhibit natural killer activity during early pregnancy. Theriogenology 2000; 54:1207–1214.
15. Martal J, Lacroix MC, Loudes C, Saunier M, Wintenberger-Torres S: Trophoblastin, an antiluteolytic protein present in early pregnancy in sheep. J Reprod Fertil 1979; 56:63–72.
16. Imakawa K, Anthony RV, Kazemi M, Marotti KR, Polites HG, Roberts RM: Interferon-like sequence of ovine trophoblast protein secreted by embryonic trophectoderm. Nature 1987; 330:377–379.
17. Roberts RM, Cross JC, Leaman DW: Interferons as hormones of pregnancy. Endocr Rev 1992; 13:432–452.
18. Spencer TE, Bazer FW: Ovine interferon-τ suppresses transcription of the estrogen receptor and oxytocin receptor genes in the ovine endometrium. Endocrinology 1996; 137:1144–1147.
19. Newton GR, Vallet JL, Hansen PJ, Bazer FW: Inhibition of lymphocyte proliferation by ovine trophoblast protein-1 and a high molecular weight glycoprotein produced by the peri-implantation sheep conceptus. Am J Reprod Immunol 1989; 19:99–107.
20. Pontzer CH, Bazer FW, Johnson HM: Antiproliferative activity of a pregnancy recognition hormone, ovine trophoblast protein-1. Cancer Res 1991; 51:5304–5307.
21. Assal-Meliani A, Charpigny G, Reinaud P, Martal J, Chaouat G: Recombinant ovine trophoblastin (roTP) inhibits ovine, murine and human lymphocyte proliferation. J Reprod Immunol 1993; 25:149–165.
22. Tuo W, MacMillan H, Gunter N, Bazer FW, Brown WC: Upregulation of interleukin-4 and IFN-gamma expression by IFN-tau, a member of the type I IFN family. J Interferon Cytokine Res 1999; 19:79–87.
23. Emond V, Asselin E, Fortier MA, Murphy BD, Lambert RD: Interferon-tau stimulates granulocyte-macrophage colony-stimulating factor gene expression in bovine lymphocytes and endometrial stromal cells. Biol Reprod 2000; 62:1728–1737.
24. Nagaoka K, Sakai A, Nojima H, Suda Y, Yokomizo Y, Imakawa K, Sakai S, Christenson RK: A chemokine, interferon (IFN)-γ-inducible protein 10 kDa, is stimulated by IFN-τ and recruits immune cells in the ovine endometrium. Biol Reprod 2003; 68:1413–1421.
25. Nagaoka K, Nojima H, Watanabe F, Chang K-T, Christenson RK, Sakai S, Imakawa K: Regulation of blastocyst migration, apposition, and initial adhesion by a chemokine, interferon γ-inducible protein 10 kDa (IP-10), during early gestation. J Biol Chem 2003; 278:29048–29056.
26. Mackay C: Chemokines: immunology's high impact factors. Nat Immunol 2001; 2:95–101.
27. Inngjerdingen M, Damaj B, Maghazachi AA: Expression and regulation of chemokine receptors in human natural killer cells. Blood 2001; 97:367–375.
28. Farber JM: Mig and IP-10: CXC chemokines that target lymphocytes. J Leukoc Biol 1997; 61:246–257.
29. Teixeira MG, Austin KJ, Perry DJ, Dooley VD, Johnson GA, Francis BR, Hansen TR: Bovine granulocyte chemotactic protein-2 is secreted by the endometrium in response to interferon-tau (IFN-tau). Endocrine 1997; 6:31–37.
30. Asselin E, Johnson GA, Spencer TE, Bazer FW: Monocyte chemotactic protein-1 and -2 messenger ribonucleic acids in the ovine uterus: regulation by pregnancy, progesterone, and interferon-tau. Biol Reprod 2001; 64:992–1000.
31. Kitaya K, Nakayama T, Daikoku N, Fushiki S, Honjo H: Spatial and temporal expression of ligands for CXCR3 and CXCR4 in human endometrium. J Clin Endocrinol Metab 2004; 89:2470–2476.
32. Moore KW, O'Garra A, de Wall Malefyt R, Vleira P, Mosmann TR: Interleukin-10. Annu Rev Immunol 1993; 11:165–190.
33. Robertson SA, Seamark RF, Guilbert LJ, Wegmann TG: The role of cytokines in gestation. Crit Rev Immunol 1994; 14:239–292.
34. Cuturi MC, Anegon I, Sherman F, Loudon R, Clark SC, Perussia B, Trinchieri G: Production of hematopoietic colony-stimulating factors by human natural killer cells. J Exp Med 1989; 169:569–583.
35. Warren HS, Kinnear BF, Phillips JH, Lanier LL: Production of IL-5 by human NK cells and regulation of IL-5 secretion by IL-4, IL-10, and IL-12. J Immunol 1995; 154:5144–5152.
36. Peritt D, Robertson S, Gri G, Showe L, Aste-Amezaga M, Trinchieri G: Cutting edge: differentiation of human NK cells into NK1 and NK2 subsets. J Immunol 1998; 161:5821–5824.
37. Campbell JJ, Qin S, Unutmaz D, Soler D, Murphy KE, Hodge MR, Wu L, Butcher EC: Unique subpopulations of CD56+ NK and NK-T peripheral blood lymphocytes identified by chemokine receptor expression repertoire. J Immunol 2001; 166:6477–6482.
38. Ashkar AA, Croy BA: Interferon-gamma contributes to the normalcy of murine pregnancy. Biol Reprod 1999; 61:493–502.
39. Ashkar AA, Di Santo JP, Croy BA: Interferon gamma contributes to initiation of uterine vascular modification, decidual integrity, and uterine natural killer cell maturation during normal murine pregnancy. J Exp Med 2000; 192:259–270.

40. Croy BA, Ashkar AA, Minhas K, Greenwood JD: Can murine uterine natural killer cells give insights into the pathogenesis of preeclampsia? J Soc Gynecol Invest 2000; 7:12–20.
41. Chaouat G, Assal-Meliani A, Martal J, Raghupathy R, Elliot J, Mosmann T, Wegmann TG: IL-10 prevents naturally occurring fetal loss in the CBAxDBA/2 mating combination, and local defect in IL-10 production in this abortion-prone combination is corrected by in vivo injection of IFN-tau. J Immunol 1995; 154:4261–4268.
42. Soos JM, Stüve O, Youssef S, Bravo M, Johnson HM, Weiner HL, Zamvil SS: Cutting edge: oral type I IFN-τ promotes a Th2 bias and enhances suppression of autoimmune encephalomyelitis by oral glatiramer acetate. J Immunol 2002; 169:2231–2235.
43. Sun H, Kundu N, Dorsey R, Jackson MJ, Fulton AM: Expression of the chemokines IP-10 and Mig in IL-10 transduced tumors. J Immunother 2001; 24:138–143.
44. Dorsey R, Kundu N, Yang Q, Tannenbaum CS, Sun H, Hamilton TA, Fulton AM: Immunotherapy with interleukin-10 depends on the CXC chemokines inducible protein-10 and monokine induced by IFN-γ. Cancer Res 2002; 62:2606–2610.
45. Tebo JM, Kim HS, Gao J, Armstrong DA, Hamilton TA: Interleukin-10 suppresses IP-10 gene transcription by inhibiting the production of class I interferon. Blood 1998; 92:4742–4749.
46. Gasperini S, Marchi M, Calzetti F, Laudanna C, Vicentini L, Olsen H, Murphy M, Liao F, Farber J, Cassatella MA: Gene expression and production of the monokine induced by IFN-γ (MIG), IFN-inducible T cell γ chemoattractant (I-TAC), and IFN-γ-inducible protein-10 (IP-10) chemokines by human neutrophils. J Immunol 1999; 162:4928–4937.